# Enzymatic Hydrolysis of Organic Phosphorus in Swine Manure and Soil

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## ABSTRACT

**Organic phosphorus ($P_o$) exists in many chemical forms that differ in their susceptibility to hydrolysis and, therefore, bioavailability to plants and microorganisms. Identification and quantification of these forms may significantly contribute to effective agricultural P management. Phosphatases catalyze reactions that release orthophosphate ($P_i$) from $P_o$ compounds. Alkaline phosphatase in tris-HCl buffer (pH 9.0), wheat (*Triticum aestivum* L.) phytase in potassium acetate buffer (pH 5.0), and nuclease P1 in potassium acetate buffer (pH 5.0) can be used to classify and quantify $P_o$ in animal manure. Background error associated with different pH and buffer systems is observed. In this study, we improved the enzymatic hydrolysis approach and tested its applicability for investigating $P_o$ in soils, recognizing that soil and manure differ in numerous physicochemical properties. We applied (i) acid phosphatase from potato (*Solanum tuberosum* L.), (ii) acid phosphatases from both potato and wheat germ, and (iii) both enzymes plus nuclease P1 to identify and quantify simple labile monoester P, phytate (*myo*-inositol hexa*kis* phosphate)-like P, and DNA-like P, respectively, in a single pH/buffer system (100 m*M* sodium acetate, pH 5.0). This hydrolysis procedure released $P_o$ in sequentially extracted $H_2O$, $NaHCO_3$, and NaOH fractions of swine (*Sus scrofa*) manure, and of three sandy loam soils. Further refinement of the approach may provide a universal tool for evaluating hydrolyzable $P_o$ from a wide range of sources.**

Phosphorus is an essential element for plant growth. Generally, it is assumed that plants take up only $P_i$ for their growth; thus, $P_o$ becomes available only after it is hydrolyzed to an inorganic form (Richardson et al., 2000; Seeling and Jungk, 1996; Tarafdar and Marschner, 1995). Organic P may constitute between 20 and 80% of the total P in surface soil horizons, with extreme values of 4 and 90% observed (Dalal, 1977). Organic P exists in many chemical forms that differ in their susceptibility to hydrolysis, and thus differ in their availability as plant nutrients.

Lack of direct methods to determine the content of $P_o$ led early investigators to apply chemical methods and chromatographic techniques to assess the types of soil $P_o$ by identification of the organic moiety of these compounds. Inositol phosphates (Caldwell and Black, 1958), phospholipids (Hance and Anderson, 1963; Stott and Tabatabai, 1985), nucleic acids (Adams et al., 1954), and other forms of $P_o$ (Cheshire and Anderson, 1975; Dalal, 1977; Steward and Tate, 1971; Wild and Oke, 1966) have been identified in soils in this way. However, this approach is laborious and is not practical for quantifying $P_o$. Phosphorus-31 nuclear magnetic resonance (NMR) offers another way to identify and quantify $P_o$ in soils (Newman and Tate, 1980). This method has identified structural features of alkali-soluble P, mainly as orthophosphate, monoester-P, diester-P, and pyrophosphate (Condron et al., 1985; Hawkes et al., 1984; Leinweber et al., 1997; Newman and Tate, 1980; Rubaek et al., 1999).

Recently, phosphatases that release $P_i$ from $P_o$ compounds have been applied to investigate the properties of $P_o$ in soils. A number of investigators have evaluated the lability of $P_o$ in soil extracts by phosphatase hydrolysis; however, the variety of enzymes used complicates data comparison (Otani and Ae, 1999; Pant and Warman, 2000; Pant et al., 1994a, 1994b; Shand and Smith, 1997). A unified approach for enzyme hydrolysis would allow data comparison across a range of $P_o$ sources and forms. Selective release of hydrolyzable $P_o$, as proposed by He and Honeycutt (2001) and Turner et al. (2002), provides a baseline for comparable characterization of hydrolyzable $P_o$. He and Honeycutt (2001) proposed to use $P_i$ released by alkaline phosphatase (AKP) to represent simple monoester P content in animal manure. Turner et al. (2002) similarly assigned AKP-released $P_i$ as labile monoester P in water-extractable soil $P_o$. Both research groups proposed that other types of $P_o$ could be represented by $P_i$ released by a relevant enzyme minus AKP-released $P_o$. A deficiency of the approach is that the incubation conditions (such as cofactors, buffer media, and pH) for AKP differ from those for other $P_o$–hydrolysis enzymes. The requirement of different incubation conditions not only makes the preparation of reaction mixtures inconvenient, but may also introduce errors due to different rates of chemical $P_o$ hydrolysis and interference by two reaction media during the P assay (He and Honeycutt, 2001; Pant et al., 1994a, 1994b). Use of a single set of incubation conditions would reduce such systematic errors.

For this purpose, we evaluated the substrate specificity of potato acid phosphatase because this enzyme has not been previously used to investigate $P_o$ hydrolysis in either soils or animal manure and it shows optimal activity at pH 4.8 and 37°C (supplier's information), close to the conditions for other phosphatases we tested previously (He and Honeycutt, 2001). We also tested the enzymatic approach, developed for animal manure $P_o$ (He and Honeycutt, 2001), in characterizing $P_o$ in soil sequential extracts where physicochemical properties

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**Abbreviations:** $CS_c$, Caribou soil with conventional cultivation history; $CS_m$, Caribou soil with manure application history; GP, acid phosphatase (Type I from wheat germ); NMR, nuclear magnetic resonance; NP, nuclease P1 from *Penicillium citrinum*; NS, Newport soil; $P_i$, inorganic phosphorus; $P_o$, organic phosphorus; PP, acid phosphatase (Type IV-S from potato); SM, swine manure; WP, phytase from wheat.

may differ from those of animal manure (He et al., 2003).

## MATERIALS AND METHODS

### Soil and Manure

Soil samples were collected from two locations. The surface (15 cm) of an uncultivated soil (unnamed series; coarse-loamy, mixed, frigid, Typic Haplorthod; 42% sand, 52% silt, and 6% clay) was collected from an area in perennial grass sod at the USDA-ARS research site in Newport, ME (NS). Soils with conventional cultivation practice ($CS_c$) and with a 10 yr history of animal manure application ($CS_m$) were collected from the surface (15 cm) of the long-term plots at the Maine Agricultural and Forest Experimental Station Farm in Presque Isle, Maine (Caribou sandy loam: fine-loamy, isotic, frigid Typic Haplorthods; 51% sand, 41% silt, and 8% clay). Soil samples were sieved (2 mm), air-dried, and stored at room temperature until use. Selected soil properties (Table 1) were measured by the Maine Agricultural and Forest Experiment Station. Modified-Morgan extraction (2 g dry soil in 10 mL of pH 4.8, 0.62 *M* $NH_4OH$ + 1.25 *M* $CH_3COOH$, shaken for 15 min) and inductively coupled plasma emission spectroscopy were used to determine soil nutrient concentrations. The swine manure (SM) collected from a local farm was homogenized, freeze-dried, ground to pass through a 0.991-mm sieve, and stored in a desiccator at −20°C until use.

### Sequential Fractionation

A modification of the method of Sui et al. (1999) was used in this study, with the extraction time in distilled water shortened from 16 to 2 h. Each sample (1.0 g of soil or 0.5 g of manure) was sequentially extracted in 25 mL of distilled water, 0.5 *M* $NaHCO_3$ (pH 8.5), 0.1 *M* NaOH, and 1 *M* HCl. Four replicate samples were fractionated. After each extraction, the tubes were centrifuged at 23 700 × *g* for 30 min at 4°C. The supernatant was passed through a 0.45-μm filter (Fisherbrand MCE membrane; Fisher Scientific, Pittsburgh, PA). Supernatant (25 mL) from the water extract of soil was freeze-dried and then redissolved in 3.0 or 3.4 mL 100 m*M* Na acetate buffer (pH 5.0) due to the low concentration of P in the extract. EDTA (1 m*M* final concentration) was added to the NaOH fraction to prevent phosphorus compounds from precipitating during pH adjustment. The $NaHCO_3$ and NaOH fractions were adjusted to pH 5.0 by slow addition of 2.5 or 8 *M* acetic acid. The $NaHCO_3$ fractions were set aside for 2 h after pH adjustment to let excessive carbonic acid ($CO_2$) bubble out.

### Enzymes

Acid phosphatases (EC 3.1.3.2) Type I from wheat germ (GP, 0.5 U $mg^{-1}$ solid) and Type IV-S from potato (PP, 5.3 U $mg^{-1}$ solid); phytase (EC 3.1.3.26) from wheat (WP, 0.03 U $mg^{-1}$ solid); and nuclease P1 (EC 3.1.30.1) from *Penicillium citrinum* (NP, 355 U $mg^{-1}$ solid) were purchased from Sigma (St. Louis, MO). NP does not directly cleave the P–O bond in $P_o$ compounds, but instead endonucleolytically cleaves polynucleotide bonds in RNA and DNA to produce mononucleotides (Webb, 1992). This was confirmed by our preliminary test in which no $P_i$ was produced from $P_o$ compounds incubated with the commercially available NP preparation. However, $P_o$ in NP-cleaved mononucleotides can be released by phosphomonoesterases (e.g., PP, GP) to produce $P_i$ (Palmgren et al., 1990; He and Honeycutt, 2001). One unit (U) of enzyme activity was defined as liberation of 1.0 μmol of relevant product from appropriate substrates at appropriate incubation conditions based on the supplier's information. It was necessary to purify WP because it possessed lower activity and $P_i$. The phytase (0.25 U $mL^{-1}$) was purified by a factor of 20 by ion exchange chromatography with Hitrap SP (5 mL) and Hitrap Q (5 mL) columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Stock solutions of PP and GP were prepared in the concentration of 10 U $mL^{-1}$ in 100 m*M* sodium acetate buffer (pH 5.0). Insoluble materials were removed by centrifuging at 23 700 × g for 30 min after the stock solutions had set aside at 4°C for 2 h. The stock solutions of WP, PP, and GP were then dispensed in microcentrifuge vials in 1 mL each and stored at −20°C until use. Nuclease P1 was purchased in 1 or 5 mg each bottle; therefore, the buffer (e.g., 0.2 mL for 1 mg NP) was directly added into the bottle to obtain an activity concentration in the range of 1700 to 900 U $mL^{-1}$ dependent on the amount and activity of NP in a specific bottle. This NP solution was stored at 4°C. A preparation of these enzyme stock solutions was generally used up in less than 4 mo.

### Enzymatic Incubation

All enzymatic incubations were performed at 37°C for 1 h in 100 m*M* Na acetate (pH 5.0) (higher buffer concentrations for $NaHCO_3$ fractions due to greater acetic acid requirement for neutralizing 0.5 *M* $NaHCO_3$). The incubation mixtures contained appropriate amounts of enzymes (GP and PP 0.25, WP 0.085, and NP 2 U per mL mixture). Precipitates that appeared in rethawed GP and PP stock solutions were removed by centrifuging for 2 min in a microcentrifuge. Controls were included whereby either the enzymes or samples (substrates) were omitted. To compare their effectiveness for releasing hydrolyzable $P_o$, the enzymes WP, GP, PP, NP, or their combinations were added to the sequential $H_2O$, $NaHCO_3$, and NaOH fractions of soils and swine manure. Soil or manure fractions were diluted to keep the concentration of $P_i$ in incubation mixtures not more than 0.3 m*M*.

To verify the effectiveness of the enzymatic classification, PP alone and combinations of PP/GP and PP/GP/NP were used to hydrolyze model P compounds. The 14 model compounds tested were phytate (inositol hexaphosphoric acid magnesium potassium salt); simple phosphomonoesters (*p*-nitrophenyl phosphate, glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, AMP, and glycerophosphate); condensed phosphates (NAD, pyrophosphate, ADP, and ATP); and polynucleotides (RNA and DNA). The concentration of each substrate except RNA and DNA was 0.1 m*M* total P. The concentrations of P in RNA from baker's yeast and DNA from salmon testes were 0.064 and 0.070 m*M*, respectively. Enzymatically hydrolyzable P was classified into three func-

**Table 1. Selected properties of Newport soil (NS) and Caribou soil with ($CS_m$) and without ($CS_c$) long-term manure application.**

| Soil | Organic matter | P | K | Mg | Ca | CEC† | pH |
|---|---|---|---|---|---|---|---|
| | % | mg $kg^{-1}$ | | | | cmol $kg^{-1}$ | |
| NS | 4.3 | 12.0 | 302 | 178 | 1149 | 4.1 | 5.7 |
| $CS_m$ | 4.5 | 37.0 | 580 | 386 | 3208 | 9.4 | 6.0 |
| $CS_c$ | 2.6 | 32.5 | 386 | 458 | 2587 | 8.3 | 5.7 |

† Cation exchange capacity.

**Table 2. Inorganic ($P_i$) and organic ($P_o$) phosphorus fractions sequentially extracted from Newport soil (NS), Caribou soil with ($CS_m$) and without ($CS_c$) long-term manure application, and swine manure (SM) by water ($H_2O$), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), and hydrochloric acid (HCl).**

| | $H_2O$ | | $NaHCO_3$ | | NaOH | | HCl |
|---|---|---|---|---|---|---|---|
| Soil/manure | $P_i$ | $P_o$ | $P_i$ | $P_o$ | $P_i$ | $P_o$ | P† |
| | mg kg$^{-1}$ dry matter | | | | | | |
| NS | 2.4 ± 0.1‡ | 2.2 ± 0.4 | 208 ± 21 | 73 ± 25 | 451 ± 17 | 239 ± 50 | 150 ± 4 |
| $CS_m$ | 18.0 ± 0.8 | 6.8 ± 1.2 | 285 ± 7 | 101 ± 5 | 1162 ± 129 | 330 ± 113 | 232 ± 8 |
| $CS_c$ | 16.8 ± 1.2 | 4.3 ± 1.0 | 295 ± 14 | 90 ± 35 | 966 ± 143 | 249 ± 21 | 220 ± 7 |
| SM | 1372 ± 251 | 511 ± 53 | 771 ± 16 | 803 ± 160 | 173 ± 15 | 235 ± 10 | 409 ± 16 |

† Only total P was measured.
‡ Mean ± standard deviation.

tional groups: simple labile monoester P (PP-released P), phytate-like P (PP/GP-released P minus PP-released P), and DNA-like P (NP/PP/GP-released P minus PP/GP-released P).

### Phosphorus Analysis

Inorganic orthophosphate (that is, $P_i$) was assayed by a molybdate blue method modified by Dick and Tabatabai (1977), with total assay volume reduced to 1 mL. It is worth noting that this method is developed for determination of $P_i$ in aqueous solution containing labile organic $P_o$ and condensed $P_i$ whereas other molybdate blue methods determine a loosely defined "molybdate-reactive P." Total P was determined in the same way after $H_2SO_4$–$H_2O_2$ digestion and adjustment to pH 5. Organic P was estimated as the difference between total P and $P_i$. With this definition, certain inorganic forms such as inorganic pyro- or polyphosphates could be in the fraction of $P_o$. No effort was made to distinguish them in this work. Enzyme-released $P_o$ was calculated as the difference between $P_i$ contents determined in the presence and absence of the enzyme(s).

## RESULTS AND DISCUSSION

### Inorganic and Organic Phosphorus Contents of Extracts

Extractable P concentration in the three soils increased with extractant strength, following the order: $H_2O$, $NaHCO_3$, and NaOH (Table 2). Another strong extractant, 1 *M* HCl, did not release more P than 0.1 *M* NaOH. This result apparently reflects the presence of less Ca-bound P (HCl-extractable) than Al- and Fe-bound P in these acid soils. In contrast to soils, most P in swine manure was present in the $H_2O$ and $NaHCO_3$ fractions, as relatively few Al-, Fe-, and Ca-oxides were found in the swine manure (He et al., 2003). Extractable $P_i$ and $P_o$ from uncultivated Newport soil (NS) were relatively low in all four fractions. Higher amounts were observed in the fractions from conventional cultivated Caribou soil ($CS_c$) and animal manure-amended Caribou soil ($CS_m$) (Table 2). The difference in P distribution in the sequential fractions between the Newport and Caribou soils was consistent with soil testing P contents in the three soils (Table 1).

### Hydrolysis of Organic Phosphorus in Sequentially Extracted Fractions of Swine Manure and Soils by Phosphatases

Although the proportion of $P_o$ released varied, similar hydrolysis patterns were observed in all fractions in the manure and soils (Fig. 1 and 2) (data for $CS_c$ and $CS_m$ not shown due to their similarity to NS). The least $P_o$ was always released by PP, indicating a relatively low concentration of simple monoester P. In most cases, similar amounts of $P_o$ were released by WP and GP. This observation supports other reports that both enzymes release similar types (Hayes et al., 2000; He and Honeycutt, 2001) and quantities (Shand and Smith, 1997)

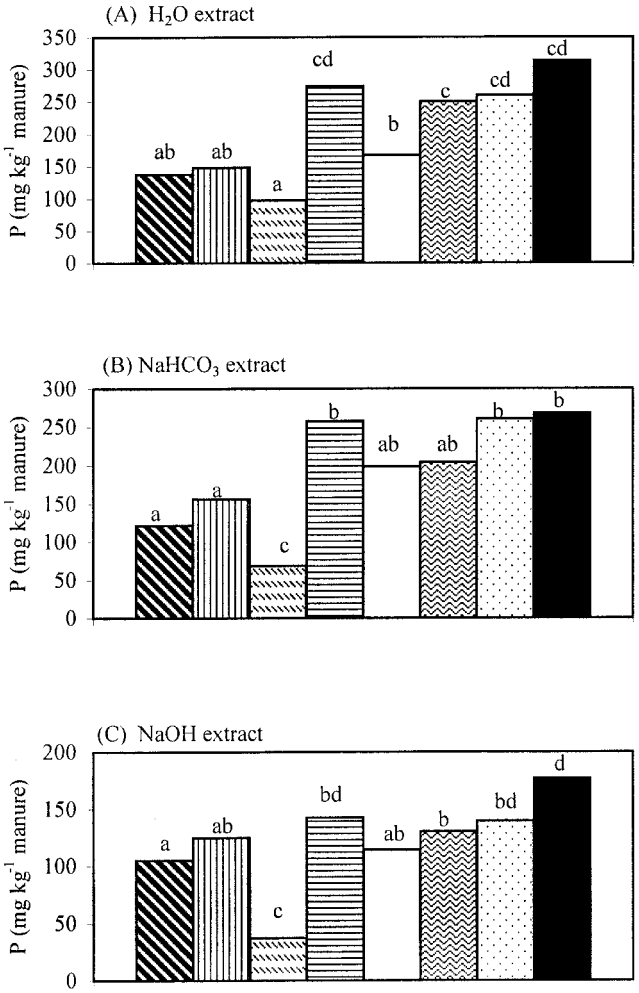


**Fig. 1. Enzymatically hydrolyzable organic P in sequential extracts of swine manure. Different letters in the same panel indicate significant difference at $P \leq 0.05$. From left to right: organic P released by phytase from wheat (WP), acid phosphatase (Type I from wheat germ, i.e., GP), acid phosphatase (Type IV-S from potato, i.e., PP), WP/GP, WP/PP, GP/PP, WP/GP/PP, and WP/GP/PP/nuclease P1 from *Penicillium citrinum* (NP).**

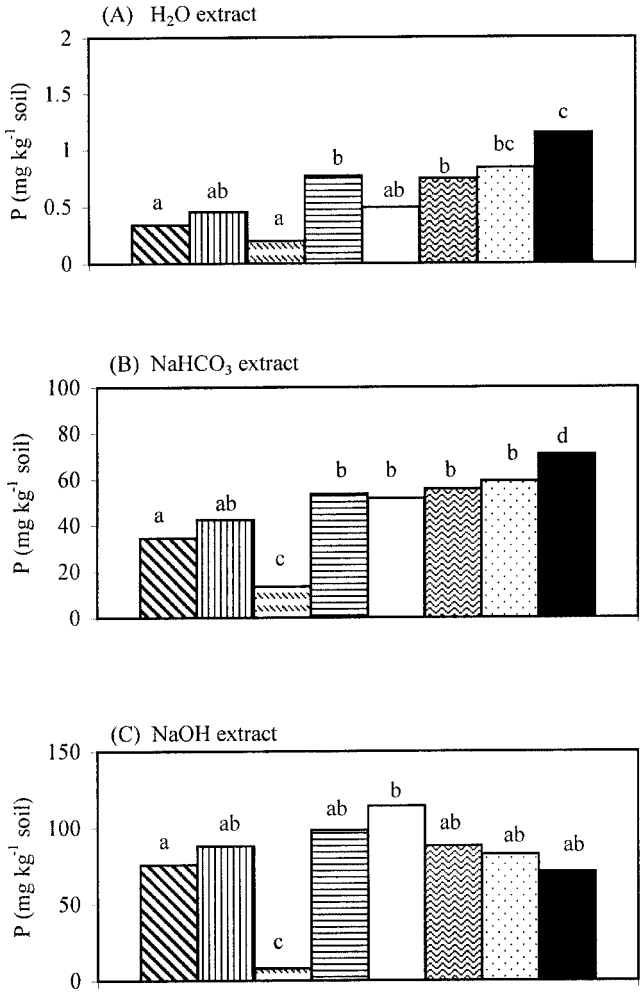


**Fig. 2. Enzymatically hydrolyzable organic P in sequential extracts of Newport soil. Different letters in the same panel indicate significant difference at $P \leq 0.05$. From left to right: organic P released by phytase from wheat (WP), acid phosphatase (Type I from wheat germ, i.e., GP), acid phosphatase (Type IV-S from potato, i.e., PP), WP/GP, WP/PP, GP/PP, WP/GP/PP, and WP/GP/PP/nuclease P1 from *Penicillium citrinum* (NP).**

of $P_o$. Combinations of the three phosphatases (WP/GP, WP/PP, GP/PP, and WP/GP/PP) generally released more $P_o$ than WP or GP alone. This indicates substrate complementarity among the three phosphate-releasing enzymes. Inclusion of NP did not significantly increase $P_o$ release in most fractions. Turner et al. (2002) reported that diester P accounted for 6 to 63% of $P_o$ in grassland soil solutions determined with enzymatic hydrolysis. In their $^{31}P$ NMR spectroscopic investigation, Leinweber et al. (1997) reported that diester P contents ranged from 0 to 73% of $P_o$ in NaOH-extracted manure and soil fractions. The diester P contents in our manure and soils were at the lower end of this distribution range.

## A Combination Approach of Enzymatic Hydrolysis for Classification of Organic Phosphorus in Soils and Animal Manure

Results of this study indicate that the enzymatic incubation scheme reported here is applicable for characterizing hydrolyzable $P_o$ from soils and animal manure. Whereas all enzymes were effective, we further tested the use of PP, PP/GP, and PP/GP/NP for the release of hydrolyzable simple labile monoester P, phytate-like P, and DNA-like P in extracts of animal manure and soils. We chose GP over WP because the commercially available GP did not require pretreatment and purification. The scheme was able to release the majority of the simple monoester P compounds in an extent to or near to 100% recovery by PP under the experimental conditions (Table 3). Neither PP nor PP/GP was efficient to hydrolyze glucose 1-phosphate. London et al. (1985) observed that a microbial phosphatase is active against a variety of four-, five-, and six-carbon sugars and sugar alcohols phosphorylated at the terminal 4, 5, and 6 position, respectively, but exhibits little or no affinity for substrates phosphorylated at the C-1 positions. Both PP and GP seemed to act in the same way. Thus, sugar 1-phosphates were regarded as unlabile in this study. Phytate-like and DNA-like P were quantitatively released by PP/GP and PP/GP/NP, respectively. Complete or partial hydrolysis of condensed phosphates by PP and PP/GP were probably due to the contaminants in the commercial PP and GP preparations as purified phosphatases show no or little activity of hydrolysis of ATP, ADP, and pyrophosphate (Lee et al., 1967; Thompson and Chassy, 1983).

It should be pointed out that this approach is at the early developing stage for $P_o$ characterization. The proposed classification was not clear-cut under the current incubation scheme with the commercially available enzymes. So we applied the words "simple" or "-like" to reflect the facts. For example, inorganic pyrophosphate could be in the group of general labile monoester P due to its high lability to monophosphatases as shown by this current work (Table 3) and other previous works (Turner et al., 2002; Shand and Smith, 1997). Glucose 1-phosphate and AMP, which were not tested by the other two groups, were not hydrolyzed quantitatively

**Table 3. Completeness of hydrolysis of P substrates (0.1 m*M* except 0.064 m*M* for RNA and 0.070 m*M* for DNA) by incubation at 37°C for 1 h with potato acid phosphatase (PP, 0.25 U mL$^{-1}$), the combination of both PP and wheat germ acid phosphatase (PP/GP, 0.25 U mL$^{-1}$ each), and the combination plus nuclease P1 (2 U mL$^{-1}$) (PP/GP/NP) in 100 m*M* sodium acetate (pH 5.0), respectively.**

| Substrate | PP | PP/GP | PP/GP/NP |
|---|---|---|---|
| | % | | |
| Phytate | −0.1 ± 0.3† | 91.9 ± 0.4 | –‡ |
| *p*-Nitrophenyl phosphate | 100.5 ± 0.5 | 100.1 ± 0.4 | – |
| Glucose 6-phosphate | 96.1 ± 1.3 | 95.8 ± 1.6 | – |
| Glucose 1-phosphate | 11.2 ± 1.0 | 18.3 ± 0.5 | – |
| Fructose 6–phosphate | 91.7 ± 0.2 | 97.1 ± 0.7 | – |
| Ribose 5-phosphate | 89.8 ± 0.7 | 91.2 ± 1.3 | – |
| AMP | 59.0 ± 1.3 | 88.8 ± 0.3 | – |
| Glycerophosphate | 92.0 ± 0.3 | 103.5 ± 0.4 | – |
| NAD | 8.2 ± 0.2 | 53.8 ± 0.3 | – |
| Pyrophosphate | 101.0 ± 0.8 | 100.3 ± 0.4 | – |
| ADP | 97.8 ± 0.5 | 100.3 ± 0.5 | – |
| ATP | 100.8 ± 0.7 | 101.0 ± 0.9 | – |
| RNA | 15.9 ± 0.7 | 45.8 ± 0.4 | 95.2 ± 0.9 |
| DNA | 2.9 ± 0.3 | 16.7 ± 0.3 | 96.0 ± 0.4 |

† Mean ± standard deviation.
‡ Not determined.

**Table 4. Hydrolyzable organic P forms present in sequential water ($H_2O$), sodium bicarbonate ($NaHCO_3$), and sodium hydroxide (NaOH) extractable organic P fractions in Newport soil (NS) and in Caribou soil with ($CS_m$) and without $CS_c$) long-term manure application.**

| P form | $H_2O$ fraction | | | $NaHCO_3$ fraction | | | NaOH fraction | | |
|---|---|---|---|---|---|---|---|---|---|
| | NS | $CS_m$ | $CS_c$ | NS | $CS_m$ | $CS_c$ | NS | $CS_m$ | $CS_c$ |
| | mg P $kg^{-1}$ soil | | | | | | | | |
| Simple labile monoester | 0.1 ± 0.0† | −0.3 ± 0.2 | 0.1 ± 0.1 | 4.8 ± 1.4 | −0.7 ± 6.1 | 21.2 ± 14.3 | 15.9 ± 6.0 | 12.2 ± 5.9 | 7.2 ± 6.0 |
| | (3 ± 0‡) | (−4 ± 3) | (3 ± 3) | (5 ± 2) | (−1 ± 7) | (20 ± 14) | (7 ± 3) | (6 ± 3) | (4 ± 3) |
| Phytate-like | 0.7 ± 0.2 | 1.0 ± 0.1 | 1.9 ± 0.5 | 64.2 ± 6.5 | 42.3 ± 23.7 | 83.3 ± 12.1 | 44.7 ± 6.0 | 68.4 ± 29.3 | 54.0 ± 38.2 |
| | (21 ± 7) | (17 ± 2) | (51 ± 13) | (71 ± 7) | (49 ± 27) | (79 ± 11) | (20 ± 3) | (36 ± 15) | (28 ± 20) |
| DNA-like | 0.3 ± 0.3 | 0.8 ± 1.0 | 0.5 ± 0.7 | 13.7 ± 7.0 | 32.1 ± 16.9 | 1.4 ± 8.5 | 3.6 ± 9.8 | 13.0 ± 5.5 | 8.6 ± 12.7 |
| | (9 ± 9) | (14 ± 17 | (13 ± 19) | (15 ± 8) | (36 ± 19) | (1 ± 8) | (2 ± 4) | (7 ± 3) | (5 ± 7) |
| Nonhydrolyzable organic | 2.2 ± 0.4 | 4.5 ± 2.6 | 1.3 ± 0.8 | 7.7 ± 8.9 | 13.2 ± 10.5 | −0.1 ± 9.8 | 160 ± 43.2 | 92.0 ± 36.0 | 121 ± 43.5 |
| | (67 ± 13) | (74 ± 42) | (34 ± 22) | (8 ± 10) | (15 ± 12) | (0 ± 9) | (71 ± 19) | (51 ± 16) | (63 ± 23) |

† Mean ± standard deviation. Values in parentheses are mean percent of total organic P in each fraction ± standard deviation.

by PP or PP/GP. On the other side, impurity of the commercial enzyme preparations yielded partial hydrolysis of non-monoester P compounds, NAD, and RNA. Further purification of these phosphatases would probably eliminate the interferences (Van Etten and Waymack, 1991; Shand and Smith, 1997).

## Distribution of Organic Phosphorus Species in Soils

We then tested the proposed approach with swine manure and soil fractions (Table 4). The portion of $P_o$ released from the swine manure is similar to that reported previously (He and Honeycutt, 2001) although the relative abundance of the three types of hydrolyzable $P_o$ changed somewhat (data not shown). In the three soil samples, 26 to 66% of $P_o$ in the $H_2O$ fraction was enzymatically hydrolyzable. Simple labile monoester P was in a range not more than 3% of total $P_o$ in the $H_2O$ fractions of the three soils. Similarly, Turner et al. (2002) observed a lower portion of labile monoester P identified by alkaline phosphatase hydrolysis. Although simple monoester P compounds are generally soluble and could be assumed $H_2O$ extractable, the low percentage in $H_2O$ fractions may reflect the fact that they had already been degraded shortly after they were released from biogenic sources due to the prevalence of monophosphatase activities in soils (Dick and Tabatabai, 1984). Phytate-like P was the major hydrolyzable form of $P_o$ in water extracts of the three soils. This observation is consistent with previous reports (Hayes et al., 2000; Pant et al., 1994a; Turner et al., 2002). The difference of phytate-like P in water extracts was significant with conventional cultivation ($CS_c$) and manure-amended ($CS_m$) Caribou soils. This could be a result of the modification of soil biochemical properties by long-term manure application practices (Parham et al., 2002).

Hayes et al. (2000) and Otani and Ae (1999) investigated the degree of hydrolysis of soil $P_o$ extracted by $NaHCO_3$. Both teams found that a small portion (1–10%) of $NaHCO_3$–extractable $P_o$ was hydrolyzable (labile) by phytase, acid, and alkaline phosphatases. Based on their observations, they questioned the assumption that $NaHCO_3$–extractable $P_o$ is labile (Bowman and Cole, 1978). In contrast, 84 to 100% of $NaHCO_3$–extractable $P_o$ in our three soils was enzymatically hydrolyzable (Table 4). This difference might be due to different soil types and management practices. However, differences in enzyme preparations and incubation strategies used might contribute to the difference, too. These observations indicate that enzymatic hydrolysis may provide information on $P_o$ bioavailability that is otherwise obscured if only based on the extractant properties, and a unified approach would be convenient for data comparison.

The enzymes hydrolyzed 29 to 49% of NaOH-extractable $P_o$ (Table 4). In the only previous report (Pant and Warman, 2000) on enzymatic release of soil $P_o$ in NaOH extracts, sandy loam soil was extracted sequentially by $H_2O$ and 0.4 *M* NaOH. As little as 0.4% and as much as 75% of $P_o$ were found to be enzymatically hydrolyzable, varying with the types of immobilized enzymes and incubation conditions. However, no quantitative specification of $P_o$ was able to be assigned by the hydrolysis strategy used by the authors. In the three soils we investigated, phytate-like P was the major hydrolyzable $P_o$ (20–36%) in the NaOH fractions, whereas simple labile monoester P and DNA-like P accounted for less than 10% each (Table 4). The common observation of the lower portion of DNA-like P in our study or diester P by $^{31}P$ NMR could be an intrinsic property of those soils or a result of chemical hydrolysis by the extractant NaOH (Leinweber et al., 1997).

It is noticeable that a considerable portion of $P_o$ extracted in the fractions was not hydrolyzed by the commercially available enzymes we used. Apparently, these unhydrolyzable P compounds were in more complex forms, such as associated with humic material (Brannon and Sommers, 1985). Thus, a hydrolysis scheme including phosphatases and enzymes that do not even directly act on a phosphoester bond may shed light on the identity of the unidentified portion of $P_o$. For example, inclusion of humic acid–depolymerizing enzymes would degrade relevant complex P compounds to simple P esters that are substrates of common phosphomonoesterases or diesterases.

## CONCLUSIONS

Phosphate-releasing enzymes can be used to investigate hydrolyzable $P_o$ in either animal manure or soils. The difference in $P_i$ determined after incubation in the presence and absence of specific enzyme(s) reflected the corresponding type and amount of hydrolyzable $P_o$ in the sample. After comparing the ability of a number

of enzymes to hydrolyze soluble $P_o$ from swine manure and three soils, we propose to use an enzymatic procedure involving acid phosphatase from potato, acid phosphatases from both potato and wheat germ, and both enzymes plus nuclease P1 to identify and quantify simple monoester P, phytate-like P, and DNA-like P, respectively, in 100 m*M* Na acetate (pH 5.0). This stepwise approach could be used to investigate hydrolyzable $P_o$ in sequentially extracted $H_2O$, $NaHCO_3$, and NaOH fractions of swine manure and soils. Further refinement of this approach may provide a comparable and universal means to investigate hydrolyzable $P_o$ from a wide range of sources.

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